# Analysis of stress induced ICS1 phosphorylation And effect of different photoperiod on MAPKs (MPK-P) activation under stress.

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Tiivistelmä – Referat – Abstract

Stress response in plants is influenced by several external and internal factors and is executed in a modular way. Environmental stimuli or stress is sensed by cellular receptors and the signal is transduced inside cell via the phospho-activation of highly conserved intracellular signaling cascades like mitogen activated protein kinase (MAPK) cascades. The signal then activates biosynthesis pathways of major stress response hormones like Salicylic acid (SA).

In *Arabidopsis* about 90% SA is synthesized via isochorismate pathway and Isochorismate synthase 1 (ICS1) is a rate limiting enzyme in this pathway. In this study, goal was to select transgenic ICS1 (homozygous) candidate lines from parent ICS1-CFP by selective regeneration. Then, by molecular and physiological characterization of transgenic ICS1-CFP plants, the function of ICS1 phosphorylation, more specifically, impact of different photoperiods (Long day; LD and Short day; SD) and stress conditions on ICS1 activity would have resolved. However, there were no homozygous candidate line from any parent ICS1-CFP plants after several screening. Nevertheless, ozone treated stress sensitivity test was performed with heterozygous ICS1-CFP candidate plants (T2 generation).

Ozone treated stress depends on stomata factor because ozone enters into plants through stomata. Therefore, stomata index analysis was performed with *sid2* and WT (Col-0) phenotypes and grown in LD and SD conditions. Since, stomata number was different between LD and SD plants of both *sid2* and WT phenotypes, a different method named Xanthine-Xanthine oxidase (X/XO) treatment was applied that induce oxidative stress regardless of stomata. Although, WT and *sid2* had shown sensitivity to the treatment, the overall cell death percentage was very low.

Lastly, our aim was to observe the impact of different photoperiods on the activation of two particular MAPKs i.e MPK3 and MPK6 under stress conditions. The phosphorylated (P-MPK3 and P-MPK6) are found abundantly in ozone treated plants as an early response. In this experiment, plants were grown in both LD and SD, stressed with both ozone and X/XO treatments, the activation of P-MPK3 and P-MPK6 was observed by protein level analysis (western blotting) in detailed time course. Although, the activation was visualized in both LD and SD plants, qualitatively the pattern was similar between day type samples and activation signal was very weak in both stress methods.

In addition, anti-ICS1 antibody provided by Agrsera ™ was tested for its efficiency to detect endogenous ICS1 protein in plants in two experimental set-up. Although the antibody could detect overexpressed ICS1-CFP protein in samples, it was not that efficient to detect endogenous ICS1 in any of the experiments.

Avainsanat – Nyckelord – Keywords

Stress response, *Arabidopsis thaliana*, ozone, MAPK, SA, ICS1

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# Abbreviations

| | |
|---|---|
| ABA | Abscisic acid |
| ARE | antioxidant response elements |
| ATP | adenosine tri phosphate |
| CBP60a | calmodulin binding protein 60a |
| CBP60g | calmodulin binding protein 60g |
| Col-0 | Columbia-0 |
| CFP | Cyan Fluorescent Protein |
| EDS1 | Enhanced Disease Susceptibility 1 |
| CAT | catalase |
| ERK's | extracellular signal-related kinases |
| GTP | Guanosine tri phosphate |
| GSH | Glutathione |
| GPX | glutathione peroxidase |
| GSTs | glutathione *S*-transferases |
| GR | glutathione reductase |
| LSD1 | lesion simulating disease 1 |
| MAP3Ks | mitogen-activated protein kinase kinase kinases |
| MAP2Ks | mitogen-activated protein kinase kinases |
| MAPKs | mitogen-activated protein kinases |
| MS | mass spectrometry |
| PAD4 | phytoalexin deficient 4 |
| OSCA1 | reduced hyperosmolality induced calcium increase 1 |
| PAD4 | Phytoalexin deficient 4 |
| PAL | phenylalanine ammonia-lyase |
| PCD | programmed cell death |
| PERK's | protein kinase-like endoplasmic reticulum kinases |
| PTMs | post-translational modifications |
| RBOH's | respiratory burst oxidase homologues |
| RLK | Receptor like kinase |

| | |
|---|---|
| ROS | reactive oxygen species |
| SAA | systemic acquired acclimation |
| SAR | systemic acquired resistance |
| SA | salicylic acid |
| SD | short day |
| IC | isochorismate |
| ICS | isochorismate synthase |
| ICS1+TP ICS1 | with transit peptide |
| ICS1-TP ICS1 | without transit peptide |
| JA | jasmonic acid |
| LD | long day |

# 1. Introduction

Plants experience changing environmental conditions throughout their lifetime in nature. Being sessile organism, plants have evolved very sophisticated and complex mechanisms in order to adopt to the environmental queues such as day length, salinity, drought, water logging, and strong wind. Although plants are well adapted to a wide range of changes in environmental queues, sometimes very rapid and harsh change leads to intensely distress condition which in turn causes various developmental defects, visible tissue damages and death (Gusta et al 2006; Dasgupta et al 1982; Ahsan et al 2007).

Stress perception and response in plants comprise a series of intracellular activities. Collectively, external stimuli triggers a series of reactions in plants i.e.- the ROS burst in apoplast (Waszczak et al 2018), activation of internal signaling mechanisms (Foyer and Noctor 2016), phosphorylation and/or de-phosphorylation of proteins (Jha et al 2017; Csaba et al 2019), signal transduction by intracellular cascades (e.g. MAPKs) (Graves et al 1999; Ahlfors et al 2004), synthesis of phytohormones, transcriptional reprogramming and establishment of redox homeostasis and programmed cell death (PCD) (Herrera-Vasquez 2015).

Plants are dependent on light, and adequate day light (photoperiod) is the main source of energy for their own food and survival. Although extended day light is suitable for reproductive growth of some plants (Koornneef et al. 1998), yet longer photoperiod can be sensed as stress in plants. Alterations in photoperiod influence the antioxidative capacities in plants during vegetative development (Becker et al 2006). Stomata are another vital factor as regulates the entry of ions or particles inside plant through leaves and provides primary defense against stress. Their role in plants immune response and stress response are well studied and reported (Melloto et al 2006; Xie et al 2010; Kangasjärvi et al 2005). Since the stomata opening and closure depend on light, photoperiod might also affect the number of stomata in plants, which in turn, affect plants response specially against ozone.

Environmental stimuli or stress is transduced inside cell by several signaling pathways in plants. Mitogen activated protein kinase (MAPK) cascades are one of those signaling pathways. Among the MAPKs, phosphorylated MPK3 and MPK6 were found after ozone exposure in plants within 30 minutes (Ahlfors et al 2004). Therefore,

P-MPK3 and P-MPK6 act as positive regulators during stress (Pitzskscke et al 2009; Chardin et al 2017). Their signaling activity in plants is well described (Rodriguez et al 2010), but how their activation pattern is influenced by different photoperiods (long day and short day) needs to be studied elaborately.

One of the important events in plant stress responses is the biosynthesis and activity of phytohormone salicylic acid (SA). SA plays vital role not only in biotic and abiotic stress responses but also throughout different developmental stages such as seed germination, overall growth, flowering and senescence (Rivas San Vicente 2011). In the model plant *Arabidopsis thaliana*, ICS1 (isochorismate synthase 1) acts as rate limiting enzyme in SA biosynthesis through isochorismate synthase pathway (Dempsey and Vlott 2009). ICS1 is a chloroplast-localized protein with a chloroplast transit peptide (TP) sequence of 45 amino acid long at its N terminus. Recently, ICS1 has been identified as an *in vivo* phosphoprotein in ozone treated plants. The identified peptide was tMtAVLSPAAAtER (T66, T68 and T77). In an earlier study to analyze ICS1 phosphorylation *in vitro*, transgenic plants carrying wild type (WT), phospho-positive and phospho-negative ICS1 proteins were generated. At first, the triple threonine (Thr) sites (T66, T68 and T77) were mutated, under CFP tag, either to alanine (A) to generate phospho-negative ICS1 construct (ECFP-ICS1-TP T66,68,77/A) or mimicked by mutating with aspartate (D) and glutamate (E) to create phospho-positive constructs (ECFP-ICS1+/-TP T66,68,77/D and ECFP-ICS1+/-TP T66,68,77/E) with or without TP (+/-TP) respectively. The SA knockout *Arabidopsis* phenotype *sid2* (salicylic acid induction deficient 2) (https://www.arabidopsis.org) were transformed with WT (ECFP-WT ICS1+/-TP), phospho-negative and phospho-positive constructs (mentioned above) and eight different groups of parent ICS1-CFP plants (heterozygous) were generated.

In the frame of this thesis, first we aimed to study the functionality of ICS1 phosphorylation by molecular and physiological phenotyping of transgenic ICS1-CFP plants. The SA knockout sid2 plants were transformed with WT, phospho-positive and phospho-negative constructs to add more parental ICS1 lines. The parent ICS1-CFP (heterozygous) plants were subjected for selective propagation to screen candidate homozygous lines. The transgenic plant lines (homozygous) are the target phenotypes and their ozone treated stress sensitivity will be tested under different photoperiods (LD and SD) along with other physiological experiments. At the second part, stomata

count was done to observe the difference of stomata index between LD and SD plants of since ozone enters in plants through stomata and the stomata activity is highly regulated by photoperiod. Later, in order to study stress sensitivity based on different photoperiods excluding stomata factor, Xanthine-xanthine oxidase (X-XO) treatment was applied as stressing method. The X-XO enzyme system is vacuum infiltrated in this process which causes immediate oxidative stress in plant samples. WT and *sid2* were the principal phenotypes in this experiment. Lastly, we aimed to study the activation of MAPKs (P-MPK3 and P-MPK6) in plants grown in different photoperiod under stress conditions. The WT (Col-0) plants were grown LD and SD conditions, and both ozone and X-XO treatments were applied as stressing methods. The level of cellular P-MPK3 and P-MPK6 was observed by western blotting within specific time points. By this experiment, the impact of different photoperiods on the activation of MAPKs under stress could be understood more pricisely.

# 2. Literature Review

## 2.1 Stress perception by plants

In their natural habitat, plants are exposed to grow and reproduce in a continuously changing environment. Since light, temperature and humidity are major stimuli that changes in daily and seasonal basis respectively, plants are very well evolved and adopted to a wide range of these stimuli. However, plants sense stress when a single or a group of environmental stimuli exceeds their tolerance level. Better understanding on how stress perception leading to the adaptation of different stress response strategy in plants, have become a main topic to the biologists and agronomist in recent years (Pandey et al 2017). Environmental stimuli are sensed by several cellular sensors and scientists have aimed at revealing plant's sophisticated stress perception and sensing apparatus for decades. One putative sensors OSCA1 (reduced hyperosmolality induced calcium increase 1) has been reported recently which can sense osmotic stress agents like high salt concentration (Yuan et al 2014). COLD1 is a putative sensor,

identified by Ma et al in 2015, which perceives chilling stress and transduce downstream signal inside cells. Both of the sensors form $Ca^{2+}$-activated permeable channels through the plasma membrane. Changes in plasmamembrane structure due to heat or cold can activate some membrane associated protein cascades like RLK's (receptor like kinases), which also act as sensor for abiotic stresses (Gish and Clark 2011). Generally, these sensors perceive the stress or stimuli as a ligand-binding module and starts the downstream activation of several signal transduction cascades. These cascades activate several transcription factors, which in turn, activates biosynthetic pathways of protein and hormones for stress response mechanisms (de Vasconcelos et al 2016, Khan et al 2018).

## 2.2 Light is the essential stimulus

Light is the most important environmental stimulus and a fundamental energy source for plants. Being sessile organisms, plants are exposed to an ever-changing environment, and changes in light intensity and quality are very crucial environmental factors influencing plant growth, development and other physiological processes. Photoperiodic changes and circadian clock are two key factors that actively affect plants vegetative and reproductive development and overall fitness which have been discussed in several studies (Stephen D. Jackson 2008; Song and Imaizumi 2010). Photoperiods also influence plants response to exogenous stresses. Increase in the intracellular ROS concentration under exogenous stress is a vital event of plants stress response mechanisms. Studies suggest that ROS production differs in plants grown in different light intensity (Niyogi K.K. 1999) and in different day length conditions (Lepistö et al 2013).

## 2.3 Ozone as a tool to study stress sensitivity

Ozone is an inorganic compound, consisting of three-oxygen atom ($O_3$) and constitutes an atmospheric element on earth. The stratosphere contains most of the ozone on earth and it prevents harmful UV lights from sun to get into earth surface. The tropospheric ozone is considered as an air pollutant with harmful effects on animal and plants. It's concentration is increasing day by day (>90ppb in some areas) due to the release of harmful oxidizing pollutant from industries around the world. Exogenous exposure of

high concentration ozone on plants and their effects are well studied and documented. Long term exposure of high concentration ozone (>150ppb) causes severe metabolic and photosynthetic disruption in cells, marking visible tissue damage in leaf as a result of programmed cell death (PCD) (Vainonen and Kangasjarvi 2014, Krasensky-Wrzaczek et al 2017). Ozone enters into plants through leaf stomata and increases the cellular ROS concentration by degrading into superoxide ($O^{2-}$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (OH-). Therefore, controlling stomata opening and closure is considered as an initial defense against ozone-mediated stress (Vainonen and Kangasjarvi 2014). Moreover, increased intracellular ROS activates a plethora of signaling networks towards various physiological and metabolic activities as well as feedback regulatory mechanisms called ROS homeostasis and scavenging through hormonal cross talk, which is also considered as a mode of ozone mediated stress response (Waszczak et al 2018).

### 2.4 ROS burst as activator of cellular signaling networks

ROS (Reactive oxygen species) are highly reactive molecules including singlet oxygen ($^1O_2$), superoxide ion ($O^{-2}\cdot$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($OH\cdot$). These active compounds are the products of different reactions inside cellular organelles like mitochondria, chloroplast and cell wall or from intracellular enzymatic reactions in peroxisomes and glyoxisomes (Desikan et al 2005, Singh et al 2016). ROS compounds, due to their highly oxidizing activity, were considered as damaging agents as they cause substantial damage by over-accumulation in the cell. However, a remarkable number of recent studies have mentioned that ROS compounds are not only important to initiate a multitude of signaling networks but also plays vital roles during plants acclimation to stress (Schmidt et al 2016). Under acute stress conditions, the increase of intracellular ROS in plants was found to influence cross talk between different cellular mechanisms in order to imply appropriate response strategy (Krasensky-Wrzaczek and Kangasjärvi 2018). ROS burst initially takes place in the apoplast of cells when plants sense stress either due to an environmental stimulus or from any exogenous element. This triggers the activation of several signaling cascades through intra cellular receptors and ion channels (Podgórska et al 2017) which in turn generates a second ROS burst inside plasma membrane initiating a complex activation network of protein cascades (Kimura et al 2017). NADPH oxidases, especially

respiratory burst oxidase homologues (RBOH's) are the key players to the production network of ROS compounds (Torres and Dangl 2005). However, due to highly reactive and oxidizing capability, ROS production and activity are highly regulated in cells by various mechanisms (Rogers et al 2016, Waszczak et al 2018). The establishment of redox homeostasis occurs enzymatically with the synthesis of enzymes like catalase (CAT), glutathione peroxidase (GPX), glutathione *S*-transferases (GSTs), glutathione reductase (GR), and peroxyredoxin as well as non-enzymatically with the effectors like ascorbate, glutathione (GSH), α-tocopherol and several flavonoids (Kapoor et al 2015, Janků et al 2019).

## 2.5 Molecular cross talk and activation of extra cellular and intracellular defense mechanisms

ROS burst in apoplast and cellular organelles like mitochondria and chloroplast initiates a multitude of signaling pathways in different stages. In order to respond to the environmental queues, plants employ either systemic acquired acclimation (SAA) or systemic acquired resistance (SAR) based on the ROS signaling depending on the stress condition (Karpinski et al 1999). The production and regulatory mechanisms of ROS interconnects different intracellular activities and influences signaling functions during different developmental stages in different tissues (Suzuki et al 2011). As a part of SAA towards heat and light stress response, the ROS wave (Miller et al 2011) of $H_2O_2$, was found to initiate within 5-10 minutes during heat stress (Suzuki et al 2013). Several studies have mentioned about the cross talk between signaling networks towards stress response. Enhanced Disease Susceptibility 1 (EDS1) is an important factor that regulates PCD against pathogens and chloroplast derived oxidative stress (Straus et al in 2010). The study also suggests that EDS1 plays a vital role as a coordinator between SA and ROS functions towards biotic and abiotic stress response. Another study by Karpinski et al in 2013 suggested the cross talk between intense light tolerance and pathogen response. The lesion simulating disease 1 (LSD1) was found to suppress the ROS-/ethylene-dependent PCD pathway by acting as a negative regulator of pathogen response genes, EDS1 and PAD4 (phytoalexin deficient 4). These two proteins together functions as multi-faceted regulatory complex and influences plants immune response signaling pathways (Rietz et al 2011).

## 2.6 Post translational modifications of proteins: Phosphorylation and de-phosphorylation towards stress signaling

Under stressed condition, ROS molecules influences several signaling pathways by the activation of phosphorylation/de-phosphorylation of some key signaling molecules, which in turn activate transcription factors and several other proteins. Protein phosphorylation/de-phosphorylation is a very common mode of post-translational modifications (PTM's) and mostly affect their structural stabilization, localization and activity pattern. Each protein has several serine (Ser), threonine (Thr) and tyrosine (Tyr) residues which act as binding sites for phosphate groups. The terminal phosphate groups from ATP or GTP are transferred to those three amino acids and several kinase enzymes catalyze these reactions (Graves and Krebs 1999). The functionality of protein phosphorylation/de-phosphorylation is switch on/switch off based in most cases. Numerous studies suggests the versatility of protein phosphorylation/de-phosphorylation network in plants stress signaling and response mechanisms (Ichimura et al 2000). For example, phytohormone ABA (Abscisic acid) signaling is strictly regulated by phosphorylatory and dephosphorylatory activities of SnRK2 and PP2A/PP2C proteins respectively (Yang et al 2017). Cellular ion channels like $K^+$ channels are also highly regulated by phosphorylation/de-phosphorylation network (Chul et al 2007). The activation of MAP kinases by phosphorylation is a well-known cellular event which is responsible for downstream signal transduction and hormonal cross talk particularly during stress signaling in plants (Fujita et al 2006).

## 2.7 Mitogen activated protein kinase (MAPK) cascades: P-MPK 3 and P-MPK 6 as active responder

MAP kinases are highly conserved kinases that establishes several signaling cascades in eukaryotes. In plants, MAP kinases transduce intracellular signals during most of the cellular processes like differentiation and growth, cell cycle, survival and death (Rodriguez et al 2010) and also transduce stress signals for a wide range of environmental queues like salinity, drought, cold, heat, ozone, UV light (Colcombert and Hirt 2008). The localization, activation and regulation, functionality of MAPKs during stress signaling has been described precisely by Bigeard and Hirt in 2018. More specifically, their signal transduction mechanisms are mostly oriented around the

synthesis, regulation and maintaining crosstalk between phytohormone network (Jagodzik et al 2018). When a stimuli is sensed by cellular receptors, a series of downstream activation/ phosphorylation starts by the activation of MAPKKK, which then phosphorylates and activates MAPKK. Sequentially, MAPKK phosphorylates and activates MAPK and, MAPK in turn activates various substrate proteins that results in the regulation of several cellular activities (Rodriguez et al 2010). Two particular MAP kinases - MPK3 and MPK6 act as positive regulators towards stress response while MPK4 as a part of MEKK1-MKK1/2-MPK4 module negatively regulates stress responses (Pitzschke et al 2009). However, MPK-s are also strictly regulated by several protein phosphatases. They are down regulated by MAKP phosphatase 2 (MKP2), a nuclear localized protein that can directly de-phosphorylate, thereby deactivate MPK3 and MPK6 and reportedly initiate stress tolerance mechanisms (Lee and Ellis 2007).

## 2.8 Synthesis of hormones and hormonal cross talk in stress response

Hormones are the biomolecules in eukaryotes that are produced in low amounts inside cells but actively regulate every stage of growth and development in eukaryots. In plants, they work as chemical messengers to maintain cellular communication and specifically coordinate signal transduction pathways towards various stress responses (Voß et al 2014). Major plant hormones that play vital roles in stress responses in plants are Abscisic acid (ABA), Jasmonates (JA), Ethylene (ET), and Salicylic acid (SA). Beside individual functions, these stress response hormones are interconnected with each other by cross talk that sophisticatedly ensures plants maximum fitness and survival strategy in adverse condition (Pieterse et al 2009). The phytohormone ABA is well known for its active role in various physiological processes like maintenance of seed dormancy, stomata closure, fruit abscission etc and specifically plants response against drought and salinity (Vishwakarma et al 2017). Along with its multitude of functional characteristics, ABA actively maintains cross talk with SA activities where ABA was found to suppress SA Induction Deficient 2, SID2 gene and thus control SA production (Yasuda et al 2008). Ethylene (ET) is another vital plant hormone that regulates the development and senescence of flower, leaves and fruits. Throughout these processes, ET maintains interactions with Auxin, Cytokinin, Gibberellin and ABA in a complex manner (Iqbal et al 2017). The intracellular JA level increases

during pathogen and harmful insect attacks on plants and plays vital role in defending plants against them (Ruan et al 2019). Interestingly, JA and SA maintains a strong antagonistic cross talk towards different stress response. This particular antagonistic relationship initiates plants defense response against pathogens and abiotic stresses at different phases (Yang et al 2019).

## 2.9 Salicylic acid: biosynthesis and regulation under stressed condition

Ortho (2)-hydroxy benzoic acid or Salicylic acid (SA) is a phenolic and lipophilic compound. In plants, SA acts as an active phytohormone and plays significant role during physiological activities like seed germination, flowering, fruit yield and transpiration in plants (Klessig and Malamy 1994, Senaratna et al 2000). Specifically, SA plays very crucial role towards both biotic and abiotic stress response mechanisms in plants. However, studies also suggest that exogenous application of SA influences tolerance against most of the abiotic stress elements in environment (Khan et al 2015). Chorismate is the precursor for SA biosynthesis and it is synthesized in two distinct pathways in plants: phenylalanine ammonia lyase (PAL) and isochorismate (IC) pathway. Phenylalanine ammonia lyase (PAL) and isochorismate synthase (ICS) are the key enzymes in those two pathways respectively. In Arabidopsis, SA is mostly synthesized through IC pathway (Vlot et al 2009) and ICS1, a chloroplast localized protein, act as a rate limiting enzyme in IC pathway (Dempsey et al 2011). In this pathway, chorismate is first converted to isochorismate by ICS1 and then isochorismate is converted to SA by isochorismate pyruvate lyase. It has a chloroplast transit sequence of about 45 amino acid long and for this reason, SA synthesis takes place in chloroplast (Strawn et al 2007). The similar pathway of ICS1 mediated SA production was also found in tobacco and tomatoes (Catinot et al 2008, Uppalapati et al 2007). Intracellular SA production and activity is dependent on ROS activity and vice versa (Wrzaczek et al 2013). Under stressed condition, the SA signaling pathway is initiated by the ROS burst, then cellular SA concentration increases which in turn activate ROS homeostasis and ROS scavenging mechanisms (Herrera-Vásquez et al 2015). However, SA level is optimally maintained through the regulation of biosynthesis inside cell as excessive SA production initiate programmed cell death (PCD) mechanisms. Regulatory mechanisms was mentioned by Seyfferth and Tsuda in 2011. The study mentions that $Ca^{2+}$ channel mediated control of SA biosynthesis

regulation can occur in two different way. It can occur either by controlling the transcription of ICS1 (isochorismate synthase 1), the rate-limiting enzyme of SA biosynthesis pathway by active transcription factor CBP60a (calmodulin binding protein 60a, homologue of CBP60g); or by inactivating EDS1 (enhanced disease susceptibility 1), that influences SA accumulation through feedback loops.

# 3. Research objectives

Within the frame of this thesis, our aim was to study molecular and physiological characterization of ICS1-CFP plants in vivo. Recent studies mostly focus on the regulatory aspects towards ICS1 mediated SA production (Wang et al 2015, Seguel et al 2018). However, the activation pattern and post-translational modifications of ICS1 is still unclear. By molecular and physiological characterization of ICS1-CFP plants, the function of ICS1 phosphorylation will be revealed. Transgenic ICS1-CFP carrying were propagated selectively for homozygous candidate lines. The candidate lines will be grown in LD and SD conditions and subjected for exogenous ozone mediated stress response evaluation. Secondly, it was necessary to take account into stomata factor in this study since ozone enters in plant through stomata and the stomata activity is highly regulated by photoperiod. Therefore, stomata number was counted to evaluate the stomata index of WT and *sid2* plants grown in LD and SD photoperiod. Then, in order to observe the stress response of irrespective of stomata factors in plants, X-XO treatment was applied as an alternative stress method. Moreover, since ICS1-CFP plants were basically the transformed phenotypes of *sid2* plants, the ion leakage results between WT and *sid2* plants, by his treatment, could provide uniformed and distinguished stress sensitivity pattern between WT and mutant plants grown in different photoperiods. Lastly, the impact of different photoperiod on the activation of cellular P-MPK3 and P-MPK6 was observed. The wild type *Arabidopsis* plants (Col-0) will be grown in LD and SD conditions, and both ozone and X-XO treatment was applied respectively. Then level of active P-MPK3 and P-MPK6 in treated samples were evaluated and the activation pattern was compared between treatments and day lengths.

# 4. Materials and methods

## 4.1 Studying ICS1-CFP plants

### 4.1.1 Growing plants for dipping

Salicylic acid knockout plants *sid2* were subjected to floral dip transformation. *sid2* seeds were first sowed into wet soil pots (peat:vermiculite 1:1), kept in dark-cold (4°C) condition for two days and then grown on 12h/12h light/dark condition for one week. Then, germinated plantlets were transplanted into new pots (5 plants/pot) and kept to grow in long day condition (16h/8h light/dark) until early inflorescences (primary bolts) appeared. Those primary bolts were removed to ensure the emergence of multiple secondary bolts with flowers.

12h/12h light/dark condition:
Light (220 μmol/second/$m^2$) 07:00 – 17:00;
Humidity 60% Day/70% Night;
Temp 23°C Day/18°C Night.

LD condition:
Light (220 μmol/second/$m^2$) 09:00 – 01:00;
Humidity 60% Day/70% Night;
Temp 23°C Day/18°C Night.

### 4.1.2 Floral dipping with Agrobacterium constructs

The Agrobacterium pre-culture was prepared by transferring a small amount from previously made glycerol stock into 5ml LB medium and growing at 28°C for 2 days in a shaking incubator. Then, 1ml from the 5ml pre-culture was inoculated into 50ml LB containing tetracycline (5mg/ml) and kanamycin (50mg/ml) and incubated again at 28°C overnight. 5ml of overnight culture was inoculated into fresh pre-warmed 300ml LB medium and incubated for 3 hours, until the $OD_{600}$ reached within 0.6-0.8. Then, cells were harvested in 300ml tubes by centrifugation at 5000rpm for 7 minutes and supernatant was discarded. The cells were re-suspended in 300ml 5% sucrose and 20μl Silvet L-77 (0.05%), mixed carefully and poured into 500ml beakers before dipping. The plants were then dipped into the bacterial suspensions and held for 5-10 seconds to ensure that all the flowers were wet. Dipped plants were then placed on their sides in the trays and covered with plastic bags. The trays were then placed in dimmed light position in a growth chamber. On the following day, plastic covers were

removed, the plants were lifted upwards and transferred to the greenhouse to grow at LD condition until seeds appear. The seeds appeared within ten days, then watering was stopped and seeds were collected separately when the plants became dry and brown.

The main objective was to screen for transgenic homozygous plants carrying the construct of interest (35S::ICS1-WT-CFP / 35S::ICS1-T/A-CFP / 35S::ICS1-T/D-CFP / 35S::ICS1-T/E-CFP). The propagation strategy was following:

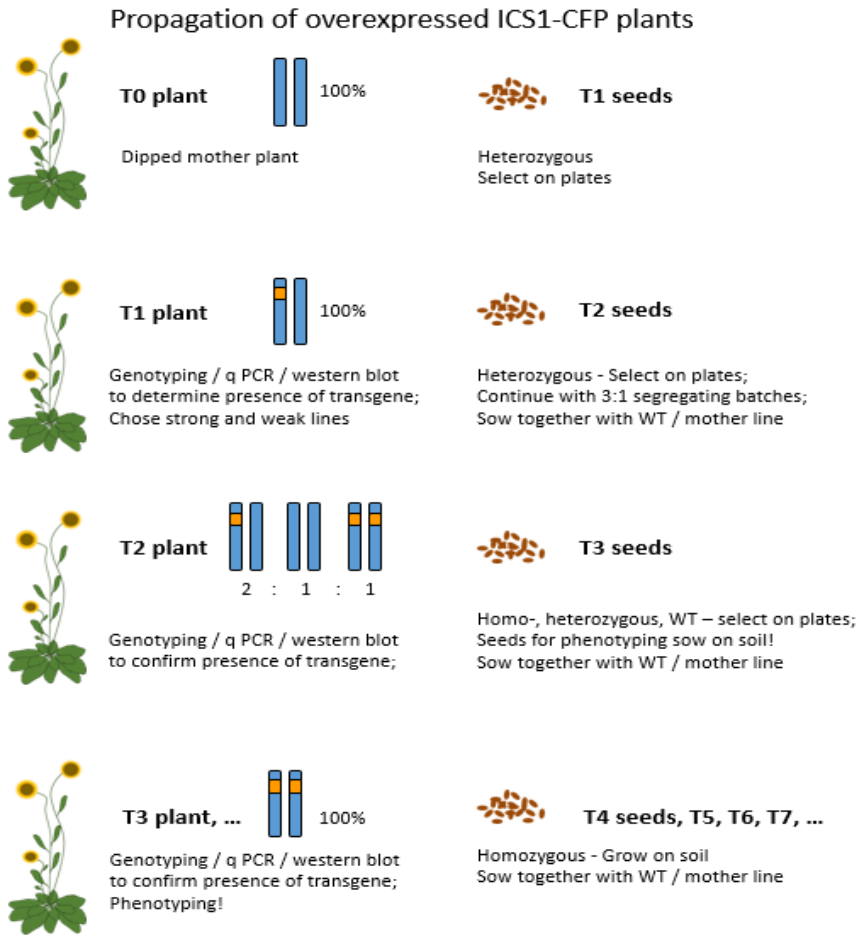



Figure 1. Overview of propagation strategy for transgenic ICS1-CFP plants: selection and regeneration.

4.1.3 Selection

4.1.3.1 Antibiotic (BASTA) selection of transformants

Seeds from each generation of ICS1-CFP plants were first surface sterilized. Approximately 50µl equivalent amount of seeds were taken into 1.5ml Eppendorf tubes, a solution of 50ml bleach: 1.5ml HCl was made in a beaker and placed in a desiccator along with the seeds, and kept for overnight sterilization with the desiccator lid closed in a chamber. On the following day, the seeds were kept in a laminar flow chamber for 2 hours to get rid of gaseous $Cl_2$, and selection plates were prepared for sowing seeds. After seed sowing, the plates were sealed with surgical tapes, kept at dark-cold condition (4$^{o}$C) for two days, and then kept in a light incubator to grow for 14 days in a condition of 23$^{o}$C: 12h light: 150µM/m$^2$/s. After 14 days incubation, successfully germinated seedlings with 2-4 green leaves and roots were identified as BASTA resistant transformants, and transplanted into soil to grow in LD condition.

Composition of Selection Medium:

4.4g/L Murashige and Skoog (MS) medium (Sigma Chemicals #M-5519:lot#SLBT2720)
5.6 g/L Phytogel (plant agar) (Sigma Chemicals #P8169 500G)
0.6 g/L (0.256M) 2-(N-morpholino) ethane sulfonic acid (MES)
pH adjusted to 5.7 with 2 N KOH
dH2O added up to 1 liter
Autoclave (120°C, 20 minutes)
0.041 M phosphinothricin/BASTA (added when the medium was cooled down after
autoclaving)

4.1.3.2 Protein level analysis of transgenic plants

A. Protein extraction with 2X SDS buffer

Protein extraction was performed during the age of 4-5 weeks when the plants had enough rosette leaves. One young rosette leaf was taken in an Eppendorf tube with glass beads and immediately frozen in liquid nitrogen. Then, frozen samples were ground in a Precellys shaking grinder and immediately 70-100µl of 2X SDS extraction buffer (according to the amount of plant material) was added, vortexed and left on ice for 15-20 minutes. The sample tubes were then heat incubated at 70°C for 10 minutes

in a shaker and then at room temperature for 20 minutes. After incubation at RT, the samples were centrifuged with 13,200 rpm for 30 minutes, the supernatant was carefully transferred to new Eppendorf tube and kept at -20°C after a snap freezing in liquid nitrogen.

B. Protein separation by SDS-PAGE

The gel casting apparatus was assembled with a 1.5mm spacer. First, the 10% separating gel was made and poured between glass plates, then overlaid with isopropanol to level up the upper surface and left at RT for 15-20 minutes to polymerize. After gel formation, isopropanol was poured off and the space was dried with Wattman paper. Then, 4.8% stacking gel was poured on top of separating gel, immediately a comb was placed and again left for 15-20 minutes to polymerize. The gel was placed together with a glass plate into the electrophoresis apparatus and filled with 1X SDS running buffer. Then, 5µl protein marker (Pageruler$^{TM}$ prestained protein ladder: pro. #26616) was loaded first and then, 20µl from sample protein was loaded into the wells. The gel was run at 30mA for 90 minutes, until the green front migrated out of the gel.

C. Western Blotting

After SDS-PAGE separation, the gels were subjected for western blotting. A PVDF (Polyvinylidene fluoride) membrane was cut to fit the gel and four pieces of Wattman paper were cut to fit membrane along with the gel. The membrane was equilibrated by a brief soaking into methanol, and Wattman papers and sponges were properly wetted with western transfer buffer before placing them into transfer cassette. The transfer sandwich was oriented as the following order: the white pole (+pole), the sponge, two Wattman papers, membrane, gel, two Wattman papers, sponge, and black pole (-pole). The transfer was carried at 65V for 1 hour with a cooling unit alongside the transfer cassette. Then, the membrane was blocked with 50ml of 5% milk in TBS-Tween solution on a shaker for 2 hours. After blocking, the membrane was transferred in a primary antibody solution (Anti-GFP Mouse IgG monoclonal antibody: Roche $^{TM}$: #11751700) at a dilution of 1:5000 in 1% milk in TBS-T solution and incubated overnight on a shaking incubator at 4°C. On the following day, the membrane was first

washed with 1% milk in TBS-T solution for 3-4 times and at the same time, the secondary antibody (Goat anti mouse: IRDye$^{R}$ 800CW: Lot#C50113-06) solution was made at a dilution of 1:7500 in 1% milk in TBS-T solution. The membrane was then incubated with secondary antibody solution in a dark LI-COR box for 2 hours on a shaker. Finally, the membrane washed 3-4 times with 1X TBS-T at room temperature and visualized using LI-COR Odyssey scanner.

## 4.2 Ozone treated stress sensitivity analysis of ICS1-CFP plants

Analysis of ozone induced stress response was performed in three stages: selection and regeneration of heterozygous ICS1-CFP plants, exposure to ozone and ion leakage measurement to ozone sensitivity.

### 4.2.1 Selective regeneration and ozone treatment of ICS1-CFP plants

Heterozygous T2 generation of ICS1-CFP plats were subjected for ozone sensitivity analysis. Seeds were first selected on BASTA plates, then germinated seedlings were transplanted and grown at LD condition (16/8 light/dark) for 18 -20 days (see details in the section 4.1.3.1). The plants were then exposed to 350ppb $O_3$ for 6 hours in a specialized chamber. After exposure, the whole rosettes were cut, immediately taken into falcon tubes containing 15-20 ml deionized water and mixed carefully by submerged swirling.

### 4.2.2 Ion leakage measurement

Ion leakage was measured using Mettler Toledo$^{TM}$ conductivity meter. After taking the ozone treated plants into water, the tubes were kept at room temperature for 2 hours. The meter was calibrated initially by setting into µS/cm unit and then by washing the sensor stick of the conductivity meter 2-3 times with clean water to keep the value near zero. Then, the sensor was dipped into the sample tubes, held until the reading reaches to a steady value, and then taken out and washed again 2-3 times. The tissue damage on the leaves done by ozone causes the ion leakage hence increases the ion level in the water which was carefully observed and recorded. After the 2 hours measurement, the tubes were sealed with caps and kept at -20$^{o}$C freezer overnight to lyse all cells and measure the ‘’total ion leakage’’. On the following day, the sample

tubes were taken out of the freezer and kept at RT until the ice melts. Then, final ion leakage was measured, and percent ion leakage was calculated with 2 hours values and total values according to the following formula:

$$\%\ ionleakage = \frac{ion\ leakage\ values\ after\ 2hours}{total\ ion\ leakage\ values} \times 100$$

4.3 Stomata index analysis by microscopy

4.3.1 Plant growth and sample collection

Stomata count was aimed to analyze if there was any difference in stomata index between Col-0 (WT) and knock out sid2 plants. Both WT and sid2 seeds were sowed to the soil, stratified in cold (4$^{o}$C) for one night and then kept at 12/12 light/dark condition for 8-10 days. Then, the germinated seedlings were transplanted into different pots and transferred into two different chambers with different daylight conditions: long day (LD: 16/8 light/dark) and short day (SD: 8/16 light/dark). The plants were matured enough to harvest samples after 18 days (LD plants) to 21 days (SD plants). Then, leaf discs were taken by punching with a borer and immediately taken into the tubes containing 7:1 ethanol/acetic acid solution. The tubes were cap-sealed and kept for 2-3 days for fixation.

4.3.2 Slide preparation and mounting

Leaf discs became white and slightly transparent after 2-3 days fixation into 7:1 ethanol/acetic acid solution. Then, the fixation solution (ethanol/acetic acid) was discarded, the discs were rinsed with water and each leaf disc was cut into two pieces where one piece was without the middle vain. With a forceps, the cut pieces were transferred on a slide keeping the abaxial side of the leaf facing upwards and one drop of Hoyer's solution was given on top of each leaf pieces. Each slide contained five pieces of discs. After placing the pieces in a good position, few more drops of Hoyer's solution were added to avoid air bubble formation. Then, a coverslip was put on the slide and after 10 minutes, extra Hoyers on the slide was removed using a paper towel. The prepared slides were then kept for 4-5 days. It is necessary to be very careful and

quick during the whole mounting process, especially when transferring and placing the discs on the slides.

Composition of Hoyer's solution: Arabic gum 7.5 g
Glycerol 5ml
Chloral hydrate 103 g
Dissolve all into 30 ml sterile water

4.3.3 Imaging and Stomata count

After 4-5 days, the discs become very transparent and ready to observe with microscope. A Leica DM2500 microscope with DIC settings was used for this study. The slides were placed on the stage of the microscope and 20X object was used to focus and visualize into the discs. The focusing was adjusted to see the stomata along with the epidermal cells. After focusing correctly, 5-7 images were taken from different positions of each sample discs. The images were then opened individually using Fiji ImageJ 1.5 software and stomata along with epidermal cells counted from each picture. The stomata index was calculated according to the following formula:

$$Stomata\ index = \frac{no.of\ Stomata}{no.of\ Stomata + no.of\ epidemal\ cell} \times 100$$

4.4 Analysis of stress sensitivity mediated by Xanthine-Xanthine Oxidase (X/XO) treatment

X/XO treatment is a direct stressing method in which an oxidizing reaction mixture is vacuum infiltrated into leaf tissue. In this method, both xanthine and xanthine oxidase are vacuum infiltrated into the leaf discs and the enzymatic reaction carries superoxides inside cells which causes cell death and tissue damage. Two different concentrations of xanthine oxidase (XO) i.e low (0.05 U/ml) and high (0.1 U/ml) were used in this experiment.

4.4.1 Growing plants and sampling

Col-0 and *sid2* plants were grown in long day (LD) and short day (SD) condition. According to the experiment plan, the plants were grown in adequate number and size to harvest nearly 250 leaf discs in total for each batch of experiment. Samples were

harvested within 18-20 days from LD plants and within 21-23 days from SD samples respectively at which age the plants reached the same size of about 10-12 leaves. A stock of 10mM Sodium phosphate buffer (pH 7.0) was prepared before sampling. For each day type plants (LD and SD), first leaf discs were taken by borer punch, immediately placed into falcon tubes with 5ml of phosphate buffer and properly mixed by swirling. There were initially six sample tubes with buffer based on the phenotypes (WT and *sid2*) and XO concentration (0/control, 0.05/low, and 0.1/high) to be applied.

10mM Sodium phosphate buffer composition: 1M (mono basic) $Na_2HPO_4$ – 2ml
1M (di basic) $NaH_2PO_4$ – 3ml
miliQ water added up to 100ml

4.4.2 Vacuum infiltration

For each sample type, 36-42 leaf discs were collected and submerged into buffer. First, a stock of 400-500 µl of 100mM Xanthine [SIGMA™: Order no. X-4002: Lot # 85H7195] in 1N NaOH was prepared and amount of xanthine oxidase [SIGMA™: Order no. X4875-40UN: Lot # SLBP5003V] for the treatments were calculated. Then, 50µl Xanthine form the stock was added to each tube and mixed properly. At the same time, 54µl suspension of xanthine oxidase (0.05U/ml) was added into two tubes as low concentration and 109µl (0.1 U/ml) was added into another two tubes as high concentration of XO, and mixed by swirling. The suspensions of XO were carefully pipetted to ensure proper mixing into the buffer-xanthine solution. Immediately, the tubes were placed in a desiccator attached with a vacuum pump, then vacuum pressure was mounted to 15-20 psi, held for 3-5 seconds and released the pressure gradually. Thus, the pressure mounting and breaking was repeated 2-3 times so that every disk was infiltrated and flocked at the bottom of the tubes. After that, the sample tubes were incubated at RT for 30 minutes to 1 hour according to the experiment plan.

Xanthine: 100mM stock in 1N NaOH (15mg in 1ml 1N NaOH)
Final concentration 1mM (50µl in 5ml buffer)
Xanthine oxidase: 4.6 U/ml stock final 0.05 U/ml (54µl in 5ml - low concentration)

final 0.1 U/ml (109µl in 5ml - high concentration)

4.4.3 Sub-sampling and Ion leakage measurement

After incubation, the buffer was pipetted out of the tubes and the discs were washed with miliQ water 3 to 4 times. Then, from each sample group, four technical replicates were made by transferring 6 discs to 15ml falcon tubes containing 5ml miliQ water. Then, ion leakage was measured using Mettler Toledo$^{TM}$ conductivity meter with similar setting and procedure as described in section 4.2.3. The measurements were taken immediately after washing (0 hour) and then at 1, 3, and 5 hour time-point. Then the tubes are kept at -20°C overnight: on the following day, they were incubated at room temperature and final ion leakage was measured as total values. Percent ion leakage was measured according to the following formula:

$$\%\ ion\ leakage = \frac{ion\ leakage\ values\ of\ a\ specific\ time\ point}{total\ ion\ leakage\ of\ the\ resp.time\ point} \times 100$$

The average percent leakage of each sample group at different time points was measured and the treatment effects were compared within the sample groups.

4.5 MAPKs (P-MPK3 and P-MPK6) activation pattern analysis

MAP kinase activation pattern analysis was performed on *A. thaliana* ecotype Col-0 plants grown in two different day light conditions i.e short day (SD) and long day (LD), treated with two different stress methods i.e ozone treatment and xanthine/xanthine oxidase (X/XO) treatment for different time points.

4.5.1 Ozone treatment, sampling and protein extraction

Col-0 plants were grown in LD and SD growth chambers at which age the plants reached the same size of about 10-12 leaves to ensure uniformed size. The plants were then subjected for ozone exposure at 350 ppb for two hours. The ozone treated samples were harvested at three different time points: 30 minutes, 1 hour and 2 hours. During each harvesting time point, one pot was quickly taken out of the chamber, the whole

rosettes were cut and taken in an aluminum foil bag and immediately frozen in liquid nitrogen. Later, the frozen samples were ground using mortar and pestle, taken into 2ml Eppendorf tubes and frozen again in liquid nitrogen. About 75-80µl of grounded powder was taken into new 1.5ml Eppendorf tubes, 75µl of Lacus buffer was added, mixed by vortexing and incubated on ice for 20minutes. Then, sample tubes were centrifuged with 13000 rpm at 4$^{o}$C for 30 minutes. The supernatant was taken into new Eppendorf tube and protein concentration was determined.

### 4.5.2 X/XO treatment, sampling and protein extraction

The X/XO treatment induces mechanical stress and its enzymatic reaction triggers superoxide production inside the cells very rapidly; hence cellular ROS level also increases immediately after infiltration. First, Col-0 (WT) plants were grown in LD and SD growth chambers and uniformed size of plants were maintained so that leaves were big enough sampling. Then, sample leaf discs were taken and subjected for X/XO treatment by following the procedure described in paragraph 4.4.1 and 4.4.2. After vacuum infiltration of sample leaf discs, the buffer was pipetted off very quickly, leaf discs were re-suspended into 15-20 ml miliQ water and transferred to a petri plate. Then, 6 discs were quickly dried in a paper towel, taken into an Eppendorf tube with glass beads and immediately frozen in liquid nitrogen. In this way, samples were prepared within six time points: 5min, 15 min, 30min, 45min, 1hour and 2hour. After sampling within expected time points, leaf discs were homogenized with the Precellys shaking machine, 75-80 µl lacus buffer was added and mixed by vortex. Then, the sample tubes were centrifuged with 13000 rpm at 4$^{o}$C for 30 minutes. The supernatant was transferred into new tubes and protein concentration was determined.

### 4.5.3 Determining protein concentration by Bradford

Bradford solution was prepared from the stock (Bio-Rad Protein Assay Dye: Reagent concentrate) by diluting at 1:5 with distilled water and adequate volume was prepared including a BSA standard. 1ml of diluted Bradford solution was taken into cuvettes for each measurements. The standard curve was set by adding BSA (1µg/ µl) with the volume of 2, 5, 7, 10, 15, 20µl. Then, 2µl of sample protein extracts was added into 1ml solution, mixed by pipetting rapidly and incubated for 15 minutes. Finally, the concentration was measured at $OD_{595}$ on the spectrophotometer.

### 4.5.4 Western blotting and detection

After determining protein concentration, sample proteins were subjected for western blotting and detection. A 12% SDS-PAGE gel was used for separation (procedure described in the section 4.1.3.2 A, B and C). The primary antibody was anti-P-MPK [Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) Antibody #9101: rabbit monoclonal] at a dilution of 1: 5000 in 1% milk in 1X TBS-T. The secondary antibody was Goat anti-rabbit [α-rabbit IgG: IRDyeR 800CW Goat] with a 1: 7500 dilution in 1% milk in 1X TBS-T.

## 4.6 Agrisera$^{TM}$ anti-ICS1 antibody testing

Choloroplastic Anti-ICS1 antibody produced in Agrisera$^{TM}$ was tested for efficiency within this thesis work. In this testing, protein extracts from Col-0, sid2 and transgenic ICS1-CFP (ICS1+T/E 4.1 – ICS1+T/E 4.4) plants were used to evaluate and compare the ICS1 detecting efficiency of anti-ICS1 (Agrisera$^{TM}$) in both endogenous and over-expressed sample protein. The test was done in two different approaches. At first, western blotting was done with sample protein from Col-0, sid2 and transgenic ICS1-CFP plants. Samples were loaded twice in a same 10% SDS-PAGE gel so that after transfer, the membrane could be cut into two separate membranes before secondary antibody treatment. The uncut membrane was treated with primary anti-ICS1 antibody (Rabbit anti-ICS1 IgG, Agrisera) with a dilution of 1:1000 in 1% milk in 1XTBS-T, and after washing, the membrane was cut into two halves. For secondary antibody treatment, one membrane was treated with Odyssey 800 –Anti-rabbit IgG at a dilution of 1:10000 in 1% milk in 1XTBS-T and another membrane was treated with Goat anti-rabbit IgG HRP conjugated with a dilution of 1:10000 in 1% milk in 1XTBS-T. In the second approach, Col-0 plants were first stressed with ozone for 3 and 6 hours, protein was extracted from those plants (including control-0h) as study suggest that ozone treatment on wild type (Col-0) plants for 6 hours increases the ICS1 transcript and activity by 4 to 5 folds (Ogawa et al 2007). The SDS-PAGE separation was done with ozone treated samples along with sid2 and one ICS1-CFP sample proteins (mICS1-T/E4 or m/E4). Western blotting was performed following the same strategy as described in previous approach. After western blotting, one membrane was visualized following Odyssey 800 protocol and another membrane was visualized by following

Amersham ECL prime Western Blotting using UVP BioSpectrum® Imaging System (LLC Upland, CA, USA).

# 5. Results

## 5.1 Selection and characterization of ICS1-CFP transgenic plants

In this thesis, our focus was to study the physiological and molecular characterization of transgenic ICS1-CFP plants in order to resolve the function of ICS1 phosphorylation. The parent ICS1-CFP plants (T1 generation) were generated by transforming *sid2* plants with four different constructs: full length ICS1 (WT), phospho-negative ICS1 mutant by Ala (ICS1-T/A-CFP), phospho-positive mimics by Asp (ICS1-T/D-CFP) and Glu (ICS1-T/E-CFP); each type with or without transit peptide (+TP/-TP). Although, some parental lines (T1 generation) were generated during an earlier study, the transformation was performed again to get more parent lines in order to screen for homozygous candidate lines from each type of ICS1-CFP plants.

Table 5.1 ICS1-CFP plants with their respective constructs

| Constructs | Transformed plant lines | Remarks |
|---|---|---|
| ECFP-WT ICS1-TP | mICS1-WT | Wild type without ICS1-transit peptide sequence |
| ECFP-WT ICS1+TP | ICS1+WT | Full length Wild type |
| ECFP-ICS1-TP T66,68,77/A | mICS1-A | Triple Alanine mutated ICS1 without transit peptide sequence |
| ECFP-ICS1+TP T66,68,77/A | ICS1+A | Triple Alanine mutated ICS1 with transit peptide sequence |
| ECFP-ICS1-TP T66,68,77/D | mICS1-D | Triple Aspartate modified ICS1 without transit peptide sequence |
| ECFP-ICS1+TP T66,68,77/D | ICS1+D | Triple Aspartate modified ICS1 with transit peptide sequence |
| ECFP-ICS1-TP T66,68,77/E | mICS1-E | Triple Glutamate modified ICS1 without transit peptide sequence |
| ECFP-ICS1+TP T66,68,77/E | ICS1+E | Triple Glutamate modified ICS1 without transit peptide sequence |

### 5.1.1 Protein level analysis of ICS1-CFP plants

After BASTA selection, transplantation and growing for four weeks, the plants (T1, T2) were subjected for protein level analysis by western blotting in order to observe the signal strength and uniformity of ICS1-CFP proteins in candidate plants respectively. The size of full length ICS1-CFP (ICS1-WT/A/D/E-CFP+TP) is 96 kDa and without transit peptide ICS1-CFP (m/WT/A/D/E-CFP-TP) is 91 kDa respectively. The candidate lines with positive and strong signal for ICS1-CFP in western blot were grown until seed harvest (T2 generation). Then harvested T2 seeds were again set for antibiotic selection followed by western blotting.

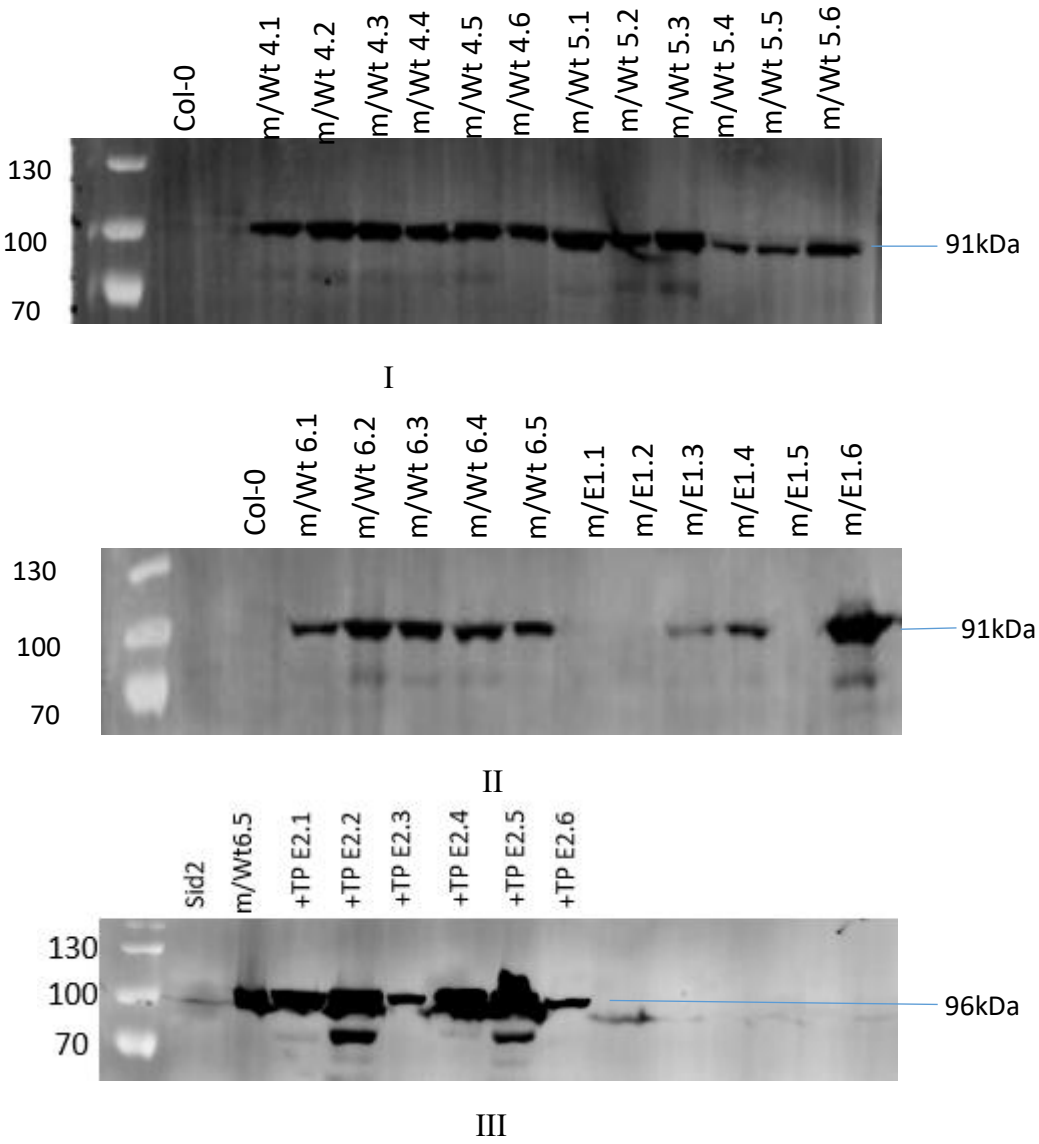



Figure 5.1 Western blotting of ICS1-CFP protein in transgenic T2 plants. (I) Strong bands at 91kDa (near 100 kDa) shows positive and uniformed signal for m/WT-TP line 4 and 5 (m/WT 4.1-4.6; m/WT 5.1-5.6), each with 6 replicates. (II) ECFP-ICS1-WT-TP line 6

also shows strong signal in all of its 5 replicates, but ECFP-ICS1-TP T66, 68, 77/E line 1 (mICS1-E) showed bands in three replicates. (III) ECFP-ICS1+TP T66, 68, 77/E line 2 (ICS1+TP E) showed strong signals at 96 kDa (near 100 kDa) in all of its replicates.

After performing western blot with T2 transgenic (heterozygous) lines, five candidate lines from mICS1-WT group, two lines from mICS1-D group, two lines from mICS1-E group and only one line from ICS1+E group appeared to be positive for ICS1-CFP protein expression (Table 5.2). However, there were no candidate T2 lines for ICS1+WT, mICS1-A, ICS1+A and ICS1+D. Then seeds (T3) were collected from those selected lines and again subjected for antibiotic selection to screen for

homozygous T3 generation. In this case, the selection plates needed to show true germination of about 100% with green plantlets. Unfortunately, no single lines appeared with expected level of true germination. The whole selection procedure was repeated again, yet there were no sign of true germination hence meaning that there were no homozygous lines from any of the transgenic plant groups. Result of propagation and selection is given in the table 5.2.

Table 5.2 List of ICS1-CFP transgenic lines and seed generations.

| **Transgenic plants** | **T1 seeds (Heterozygous)** | **T2 seeds (Heterozygous)** | **T3 seeds (Homozygous)** |
|---|---|---|---|
| mICS1-WT | 1 to 13 | 4,5,6,8,12 | - |
| ICS1+WT | 2 to 11 | - | - |
| mICS1-A | 1 | - | - |
| ICS1+A | 1,2,3,4 | - | - |
| mICS1-D | 1,2,3,4,5 | 2,5 | - |
| ICS1+D | 3,5 | - | - |
| mICS1-E | 1,2,3,4,5 | 2,4 | - |
| ICS1+E | 1,2,3,4 | 4 | - |

### 5.1.2 Ozone sensitivity analysis of ICS1-CFP plants

As a part of physiological characterization, ozone sensitivity was analyzed to evaluate the effect of ICS1-CFP plants. In general, *Arabidopsis spp* are facultative long day plants. Moreover, there were no homozygous candidate plants from any of the transgenic plant groups. Therefore, some candidate lines (m/WT 8.2, m/WT 12.5, ICS1+E 4.2, m/E4.3, m/D2.5, m/D 5.3) from heterozygous T2 generation were

selected and grown in LD condition only. The plants were subjected for ozone exposure followed by ion leakage measurement.

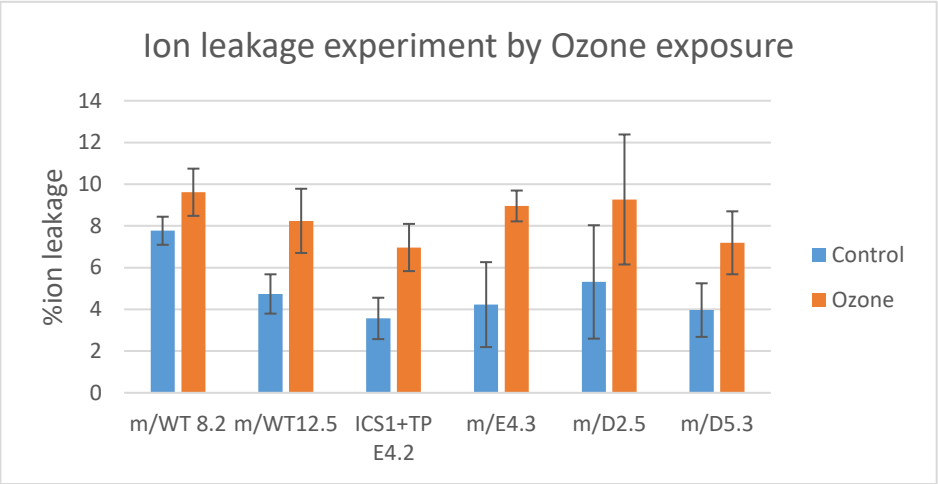



Figure 5.2 Percent ion leakage of candidate T2 (heterozygous) ICS1-CFP plants after ozone exposure.

After ozone treatment, the percent ion leakage of each candidate lines was measured and the treatment effect was compared along with their controls respectively (Figure 5.2). In order to observe significant variation of percent ion leakage pattern between lines, Analysis of Variance (ANOVA; two factor without replication; $\alpha = 0.05$) was performed. The ANOVA summary suggests that (Appendix II A) percent ion leakage values were significantly high to the ozone treated samples compared to their controls ($p < 0.05$) for each candidate lines respectively. Although the values were different between lines, collectively ion leakage pattern was considerably low (<10%) for each candidate lines. The experiment was executed two more times, yet the overall ion leakage patterns were consistently low and not uniformed between lines. Therefore, it can be said that although the transgenic lines were sensitive to ozone treatment, the overall cell death pattern were very low, and not very precise and significant enough in terms of ozone treated stress sensitivity.

5.2 Stomata index analysis by microscopy

Ozone induced stress response greatly depends on stomata since ozone enters leaves through the stomata. ICS1-CFP plants were basically transformed with SA knock out sid2 plants. Therefore, we decided to observe if there was any difference in stomata number between wild type and sid2 plants grown on LD and SD condition.

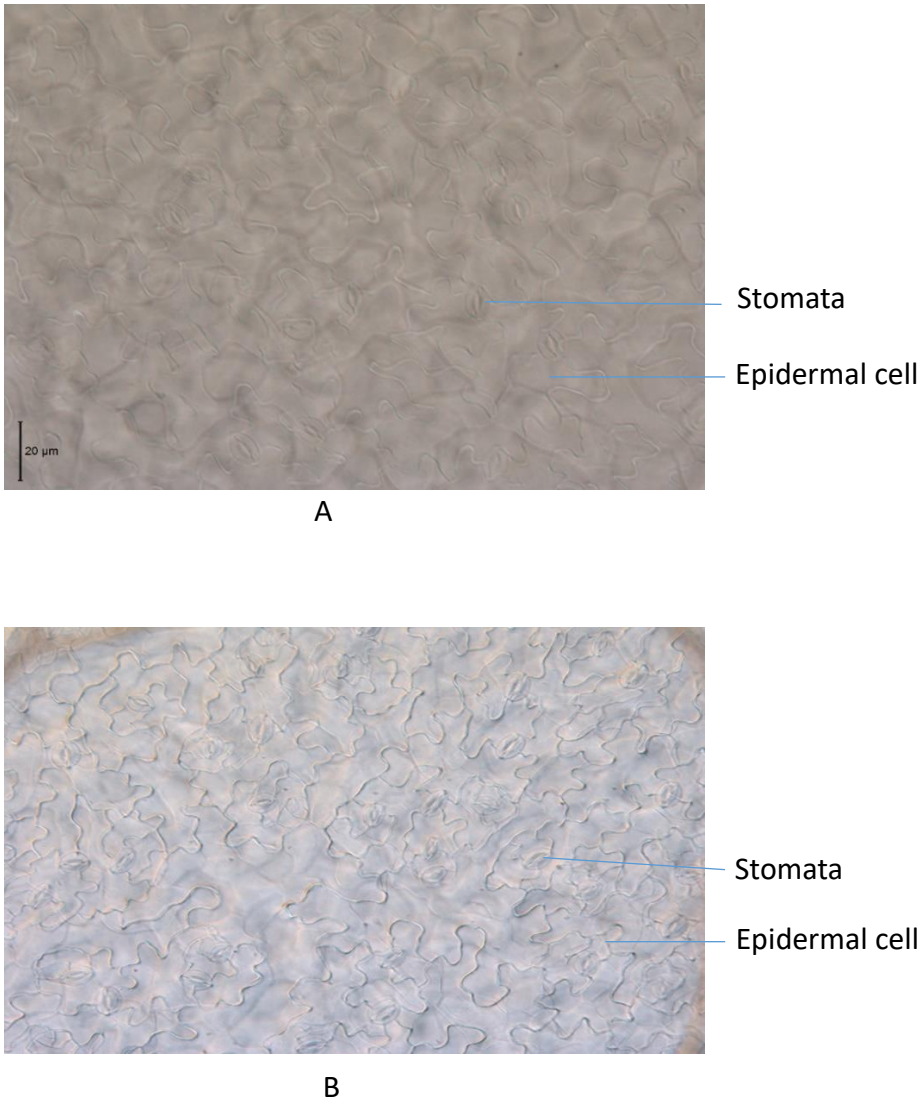



Figure 5.3 Microscopic view of stomata and epidermal cells in a sid2 plant grown in LD condition (A) and picture B was from a wild type (WT) plant grown in SD condition.

After taking pictures of WT and sid2 plants from both LD and SD conditions, number of stomata and epidermal cells were counted using Fiji ImageJ 1.5 software and the stomata index was calculated according to the following equation:

$$Stomata\ index = \frac{no.of\ Stomata}{no.of\ Stomata + no.of\ epidemal\ cell} \times 100$$

Table 5.3 Stomata index showing the comparative number of stomata in WT and sid2 grown in LD and SD condition

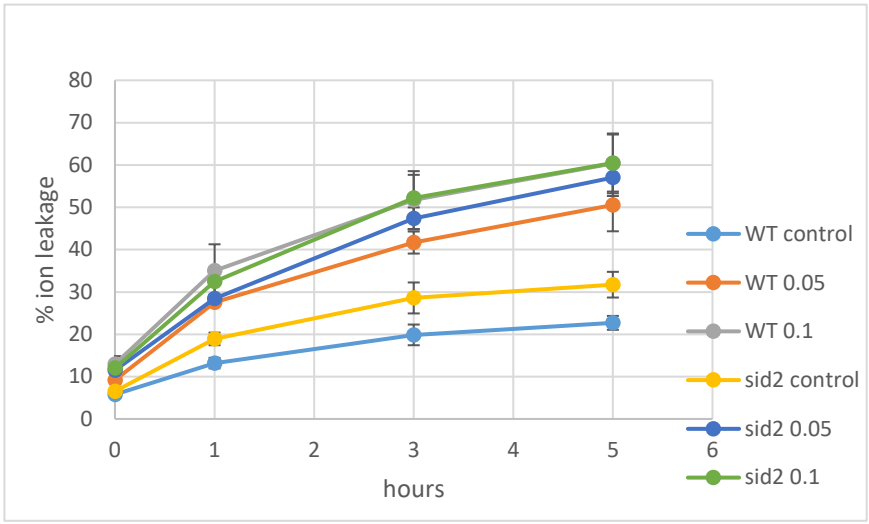
| Plant type / Day type | Long Day | Short Day |
|---|---|---|
| Wild type (Col-0) | 27.0523 ± 0.9213 | 23.6018 ± 0.2819 |
| sid2 | 26.5055 ± 0.7491 | 23.0723 ± 0.6881 |

Stomata index suggests that both WT and sid2 plants grown in LD condition had more stomata than SD condition.

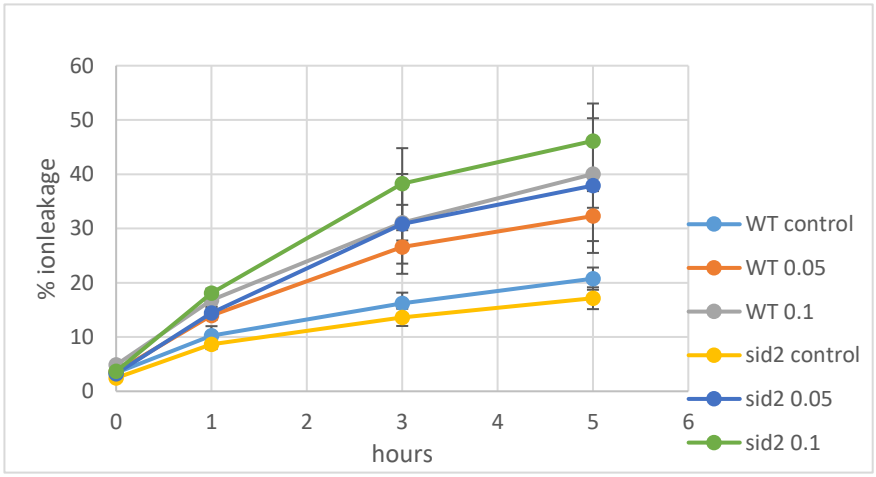
## 5.3 Xanthine-Xanthine oxidase (X/XO) treated stress response analysis

The stomata index suggested that the number of stomata were different between LD and SD type plants for both WT and sid2; hence, it was necessary to follow a different stressing method in order to observe the stress sensitivity difference between those two phenotypes and day type (LD and SD) plants. Therefore, X/XO treatment was applied in which oxidative stress is induced by vacuum infiltration of the enzyme system Xanthine-xanthine oxidase inside leaf discs. In this experiment, two different concentrations of XO, named as low (0.05 $Uml^{-1}$) and high (0.1 $Uml^{-1}$) was applied. The stress sensitivity was evaluated by measuring percent ion leakage or percent cell

death within five hour time period (1hour interval) and compared within the phenotypes (WT and *sid2*) and day lengths (LD and SD).




LD plants




SD plants

Figure 5.4 Ion leakage measurement by xanthine-xanthine oxidase (X-XO) treatment in LD and SD plants. WT/sid2 0.05 = samples treated with XO concentration of 0.05 U/ml (low). WT/sid2 0.1 = samples treated with XO concentration of 0.1 U/ml (high).

The percent ion leakage values were subjected for ANOVA (two factor without replication; $\alpha = 0.05$) to observe and analyze significant differences in the cell death pattern between LD and SD condition plants (Appendix II; B1 and B2). The ANOVA summary suggest that the ion leakage pattern for both low and high concentration

treatments were significantly higher than their respective controls for both WT and *sid2* plants in LD and SD conditions respectively (* = $p << 0.05$, significantly different; B1 and B2 ANOVA). However, the numerical difference between low and high concentration treatments effect on WT and *sid2* phenotypes were very narrow when were compared to each other (WT 0.05 to *sid2* 0.05; WT 0.1 to *sid2* 0.1). In LD condition at fifth hour incubation time point, both WT (gray) and sid2 (green) samples had 60% ion leakage at high conc. XO (0.1U/ml). At low conc. XO (0.05U/ml), sid2 samples had more than 55% ion leakage and WT had 50% ion leakage after five hours. Therefore, in LD condition, it was difficult to differentiate the sensitive phenotype to X-XO mediated stress.

On the other hand, in SD condition at high conc. XO, sid2 plants had 45% cell death whereas WT plants had 40% cell death. At low conc. XO, sid2 plants had 38% ion leakage and WT plants had 32% ion leakage. Apparently, sid2 SD plants were sensitive than WT LD plants to some extent in this method. The experiment was executed two more times and the cell death pattern remained consistent between 40-60% for both WT and sid2. Although sid2 plants showed slightly more sensitivity than WT plants in SD condition, overall cell death pattern was consistently low and robust between treatments and replication.

### 5.4 Stress induced activation pattern analysis of MAPKs (MPK3 and MPK6)

In this stage of study, our focus was to observe the effect of different photoperiods on the activation pattern of MAPKs under stress in terms of evaluating cellular active P-MPK3 and P-MPK6 levels. As explained earlier, the experiment was done according to the following criteria; 1. grown in LD and SD condition, 2. stressed with ozone and X-XO treatment within specific time points.

#### 5.4.1 MAPKs activation analysis by ozone ($O_3$) treatment

Plants grown in LD and SD condition, exposed at 350ppb ozone for two hours and sampling was done at 30 minutes, 1 hour and 2-hour time points. The samples were harvested from both control and ozone treated WT plants in LD and SD conditions.

Then, western blotting was performed to observe the level of active P-MPK3 and P-MPK6 in sample proteins LD and SD plants.

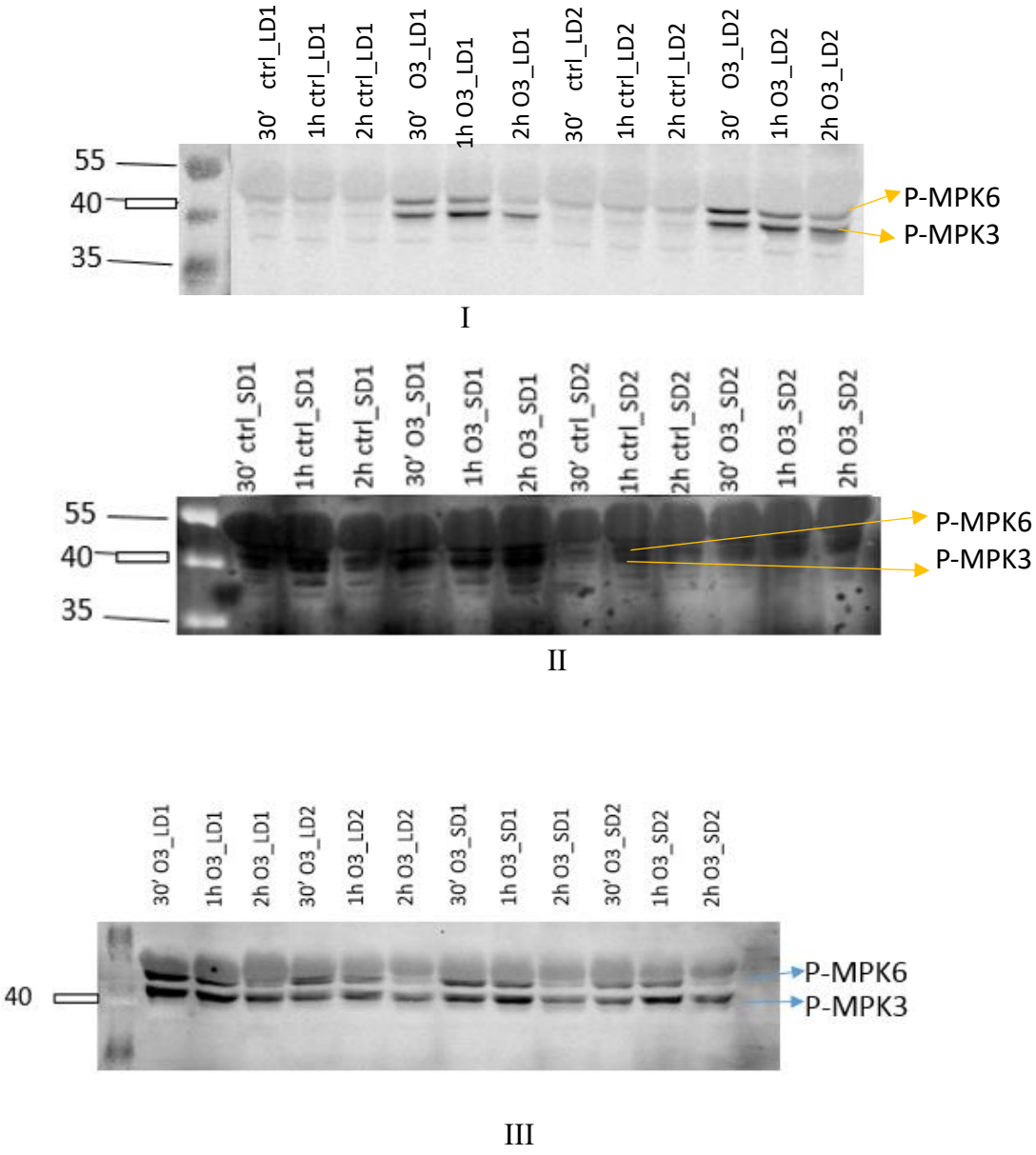


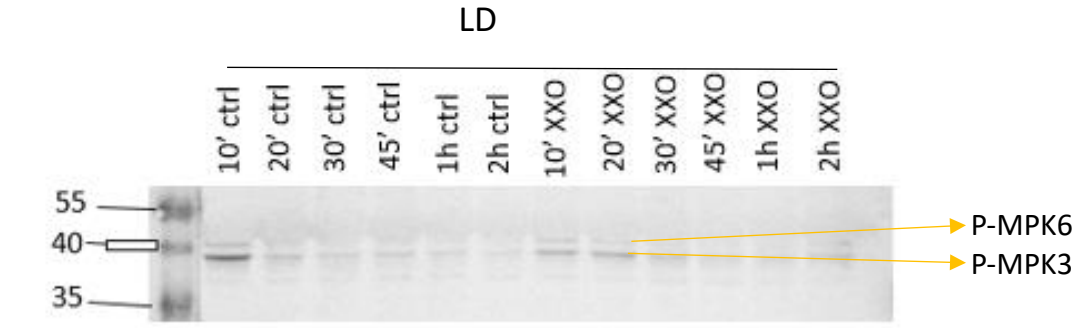

Figure 5.5.1 Ozone induced activation of P-MPK3 and P-MPK6 in LD and SD plants at 30 minutes, 1 hour and 2 hour time points. (I) long day replicates (LD1 and LD2) with controls. (II) SD replicates with controls. (III) All LD and SD samples in one gel.

The western blotting results clearly showed that ozone treatment activated the MPK's (MPK6 and MPK3) over 2 hours of exposure in both LD and SD samples. The

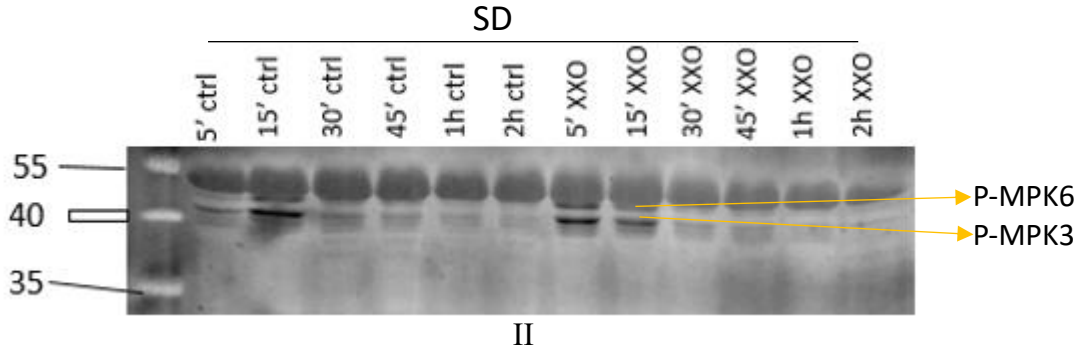
activation intensity was very high at 30 minutes samples then gradually decreased at 2 hours, and activation pattern in LD and SD plants were also qualitatively similar.

5.4.2 MAPKs activation pattern analysis by Xanthine-Xanthine oxidase (XXXO) treatment

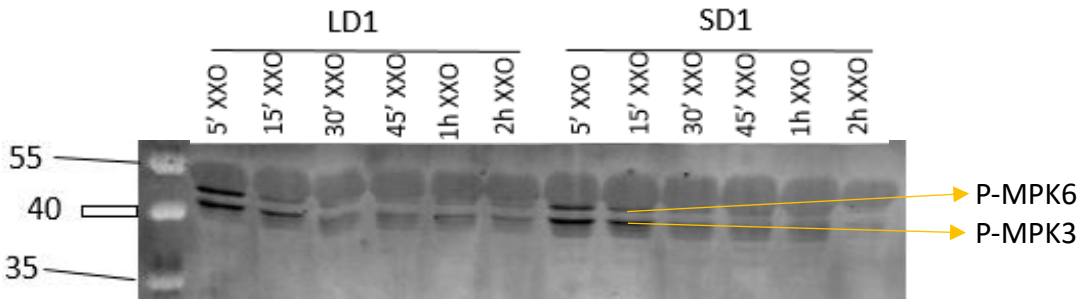
After X/XO treatment, the leaf discs were incubated at RT over 2 hours and infiltrated discs were transferred into 1.5ml Eppendorf tubes at six different time points: 10minutes, 20minutes, 30 minutes, 45 minutes, 1 hour and 2 hours to observe the activation pattern within more detailed time points.




I




II




III

Figure 5.5.2 Western blotting with X/XO treated samples to analyze activation of MPK-6 and MPK-3 in at six different timepoints. (I) Active P-MPK3 and P-MPK6 in LD samples with controls. (II) Active P-MPK3 and P-MPK6 in SD samples with controls. (III) Active P-MPK3 and P-MPK6 in LD and SD samples.

It is apparent from the blotting picture (figure 5.5.2) that the activation signal was visible only within 15-20 minutes of treatments; then disappeared in the longer treatment time points (30 minutes, 45 minutes onwards) suggesting that the X/XO samples did not show P-MPK3/6 activation. However, earlier replication of this experiment shown very strong and clear signal of activation at 1 hour and 2 hours treatments.

5.5 Agrisera$^{TM}$ anti-ICS1 chloroplastic antibody testing

Anti-ICS1 antibody produced in Agrisera$^{TM}$ was tested in two stages. In first stage, there were very strong signals of ICS1 in transgenic ICS1-CFP candidate protein ICS1+T/E4.1-4.4 (+TP T/E4.1-4.4) in Odyssey 800-Anti-Rabbit IgG treated membrane and Goat anti-rabbit IgG HRP conjugated antibody treated membrane respectively. However, the signal intensity was same in both membrane and there were no signal from Col-0 sample protein in any of the membranes (Figure 5.5.1). It is apparent that the Agrisera anti-ICS1 antibody could detect the over-expressed ICS1 protein in the sample but not the endogenous ICS1 in wild type.

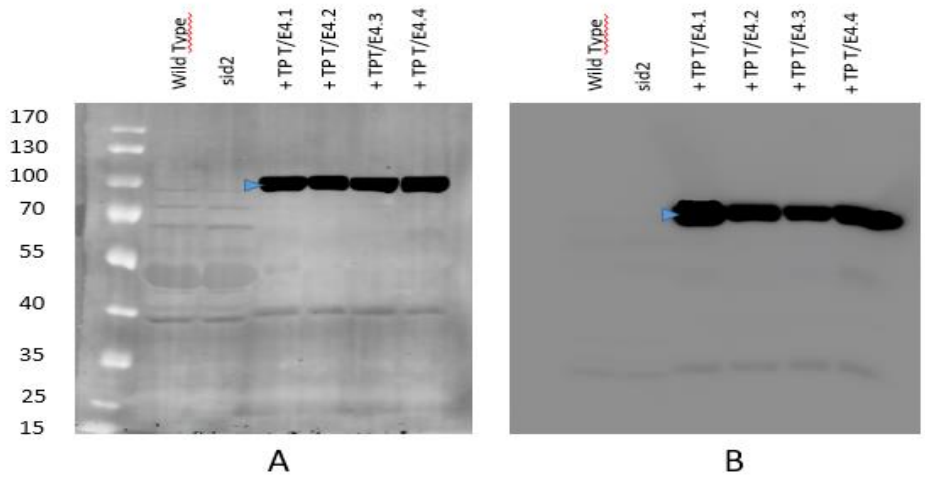


Figure 5.6.1 Western blotting to test anti-ICS1 choloroplastic antibody from Agrisera$^{TM}$. A. Membrane treated with Rabbit IgG-α-ICS1 (Agrisera$^{TM}$) as primary antibody and Odyssey 800 –Anti-rabbit IgG as secondary antibody. B. Membrane treated with Rabbit IgG-α-ICS1 (Agrisera$^{TM}$) as primary antibody and Anti-rabbit IgG HRP conjugated (Agrisera$^{TM}$) as secondary antibody. Blue arrow indicates the strong signals for ICS1-CFP candidate proteins +TP T/E 4.1-4.4. No signal found for Wild type (Col-0)

One study by Ogawa et al in 2007 suggested that the ICS1 expression level is remarkably increased in plants exposed to ozone for three hours. Therefore, in the second stage, the ozone treated samples along with sid2 and candidate ICS1-CFP sample protein m/E4 (mICS1-T/E4) were subjected for western blotting and detection by both methods. This time there was strong signal for ICS1 in m/E4 sample in both membranes but no signal appeared in ozone treated samples in Odyssey 800 membrane. Endogenous ICS1 has a molecular weight between 64-69 kDa. There was a very vague signal near 70kDa in ozone treated Col-0 samples, which also appeared in *sid2* sample, is basically a background signal (figure 5.5.2 blue arrow) in anti-ICS1 Agrisera membrane. Hence, the efficiency of Agrisera anti-ICS1 antibody in binding endogenous ICS1 is not satisfactory.

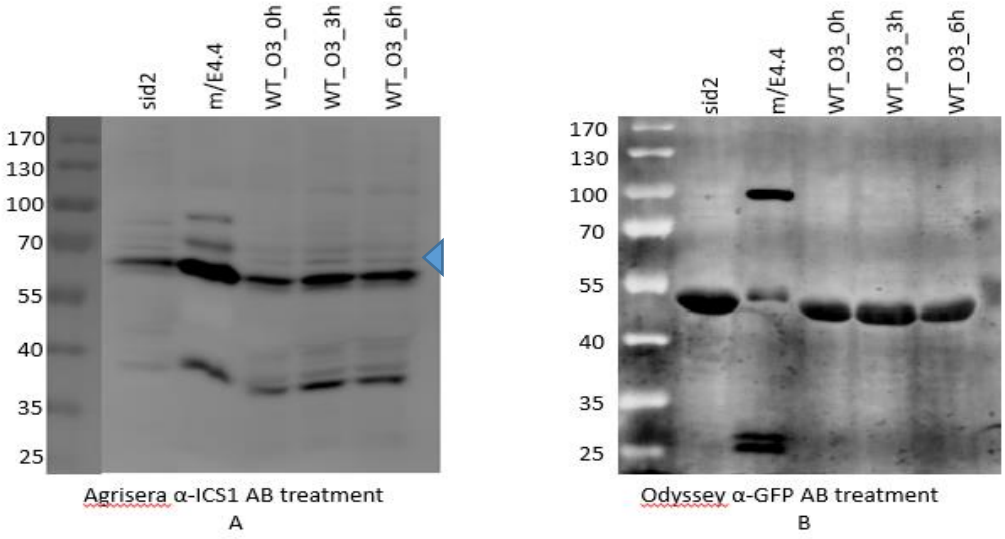



Figure 5.6.2 Western blotting to test anti-ICS1 choloroplastic antibody from Agrisera$^{TM}$. +TP T/E4.1 and WT_O3_0h to 6h = positive controls, sid2= negative control. A. Membrane treated with Rabbit IgG-α-ICS1 (Agrisera$^{TM}$) as primary antibody and Anti-rabbit IgG HRP conjugated (Agrisera$^{TM}$) as secondary antibody. B. Membrane treated with Rabbit IgG-α-ICS1 (Agrisera$^{TM}$) as primary antibody and

Odyssey 800 –Anti-rabbit IgG as secondary antibody. Blue arrow indicates the position of desired protien.

# 6. Discussion

6.1 Studying ICS1-CFP plants: Molecular and physiological experiments

In frame of thesis work, our initial objective was to study the molecular and physiological phenotyping of ICS1-CFP plants in order to understand more about the significance of ICS1 mediated SA production in plants grown under different day lengths. Earlier, ICS1 has been successfully identified by mass spectrophotometry as an *in vivo* phosphoprotein in ozone treated WT (Col-0) plant grown in short day (SD) condition (Krasensky-Wrzaczek 2018 unpublished). The detected phosphorylation sites were threonines 66, 68 and 77 (T66, T68 and T77). This study includes the generation of transgenic plants carrying WT ICS1 protein with/without transit peptide (under 35S promoter: cloned into binary vector pGreenII0229-35S-ECFP), and also plants carrying mutated phospho-sites with either alanine, aspartate or glutamate (ECFP-WT ICS1+/-TP T66, T68 and T77/A/D/E). In this way, the parent transgenic lines (T0) of ICS1-CFP plants has been established and selective regeneration was conducted with the offspring T1 and T2 (heterozygous) generation seeds to get homozygous ICS1-CFP candidate plants (T3). Each type of parent transgenic plants had their offspring in T1 and T2 generation, but no candidate plants in T3 generation could be selected as homozygous. This may be due to different reasons: one of the possibility can be the use of 35S promoter. The 35S promoter might be too strong for the transcripts as it is originated from CAMV virus. Therefore, 35S promoter could have suppressed the expression of the transcripts. Only successful transformation was reported by Strawn et al. (2007) so far which indicates that the cloning was performed with a native ICS1 promoter (full length 2.5kb).

Although there were no homozygous T3 candidates of ICS1-CFP plants identified, ozone sensitivity was tested with candidate T2 heterozygous plants. However, data analysis suggested that the candidate plants was very less sensitive to ozone treatment; therefore, it was not possible to say how transformation influenced the stress

sensitivity. Furthermore, it is possible that the plants became primed for stress because they were grown on antibiotic selection plates.

### 6.2 Stomata factor and perspectives of stress experiment by X/XO treatment

In this thesis work, we aimed to explore plants stress response under different photoperiod in terms of elaborative study on the functionality of ICS1 phosphorylation. The ICS1-CFP plants, the subject phenotypes in this study, are the overexpressed *sid2* plants carrying ICS1-CFP constructs. However, the ozone treated percent ion leakage analysis could not provide any significant stress response pattern for those plants. Therefore, we assumed that the stomata could be an interfering factor in this regard; because ozone treated stress response depends on stomata number in leaf since ozone enters into leaves through stomata. Plants physiological activities are greatly influenced by stomata. Earlier, water loss activity was observed on WT (Col-0) and *sid2* phenotypes grown in LD and SD condition. Significantly, *sid2* plants were found to loose water faster than WT plants in LD condition and for both WT and *sid2* plants, and LD plants loose more water than SD plants. Considering the facts mentioned above, we performed microscopy with WT and *sid2* plants to evaluate the stomata index of these two phenotypes grown in LD and SD condition in order to observe if stomata number was affected by phenotypic difference or photoperiod. The stomata index results suggests that the LD plants had more stomata than SD plants, but in between phenotypes, stomata number was quite same, which indicates that the number of stomata can vary due to difference in growth condition.

Therefore, in order to study stress response in plants based on different day lengths or photoperiods and irrespective of stomata factor, X/XO treatment was applied as stressing method. The same phenotypes WT and *sid2* grown in LD and SD conditions were subjected for X/XO treated stress sensitivity analysis. The ion leakage data analysis suggested that the sid2 plants were slightly more sensitive to the stress compared to WT plants in each treatment types and day conditions respectively although the overall percent cell death pattern was consistently low and robust between treatments and experimental replications. The *sid2* phenotype lacks SA synthesis capacity due to a point mutation in ICS1 gene which can be the reason of their increased sensitivity to X/XO stress. However, there might be some factors that affected the efficiency of X/XO method such as; sample plants age, incubation time

after infiltration or less active enzyme reaction. Nevertheless, the X/XO is a potential method to observe precise stress sensitivity pattern on purpose if executed with enough repetition and proper troubleshooting.

### 6.3 MAPKs activation pattern analysis in plants: different growth and different stress conditions

Plants stress response is significantly dependent on the activities of intracellular signaling pathways like MAPK cascades and their activation is mainly phosphorylation dependent. Since we were studying the functionality of ICS1 phosphorylation, additionally analyzing the activation of cellular MAPKs (P-MPK3 and P-MPK6; abundantly visible in stressed plant and act as early responder to stress) under different photoperiods and stress could have provided valuable insights towards plants stress response. Therefore, in this part of the study, the cellular level of phosphorylated P-MPK3 and P-MPK6 was observed in LD and SD plants (Col-0) stressed with ozone and X/XO treatment at different time points in order to understand the impact of different photoperiods on MAPKs activation pattern more precisely under stress. However, in case of ozone treatment, there were no distinguished activation pattern of MAPKs between LD and SD plants since cellular active P-MPK3 and P-MPK6 level was visually similar in both day length plants. Earlier experiments suggests that longer and stronger signal for active MPK's were found in LD plants than SD plants. Sampling age of the plants could have been a reason for such deviation from earlier results. In this regard, quantitative analysis with more repetition may establish a precise interpretation on how photoperiod affect the MAPKs activation under ozone stress.

Although the X/XO treatment was not effective during stress sensitivity study, it was reconsidered as stressing method in MAPKs activation study since earlier experiments suggests evidence of strong signal for P-MPK3 and P-MPK6 in X-XO treated samples. The sampling was done at six different time points i.e. 5min, 15 min, 30min, 45min, 1hour and 2hours to observe the activation signal of MPK's in LD and SD plants in a more detailed time course. Unfortunately, the activation signals were very weak and disappeared after 30 minutes of incubation. One possibility is that may be the enzyme reaction was not strong and stable enough to induce intracellular damage in the discs.

## 6.4 Testing Anti-ICS1 antibody from Agrisera™

Anti-ICS1 antibody provided by Agrisera™ was tested for its specificity against both endogenous ICS1 and transgenic ICS1-CFP. The testing was performed in two stages. At first stage, the anti-ICS1 antibody was tested alongside anti-GFP antibody with sample proteins from WT, sid2 and ICS1-CFP plants by western blotting and visualization. The strong signal of ICS1-CFP proteins in both membranes, particularly in anti-ICS1 (Agrisera™) antibody treated membrane, suggests that the antibody can detect ICS1 in transgenic plant samples. However, there were no signal of ICS1 in WT sample in Agrisera antibody treated membrane; hence, its efficiency in binding exogenous ICS1 is not clear yet. It may happen due to the sample quality; may be the crude protein concentration was too low. In second stage, ozone treated samples were tested with both antibodies, and one ICS1-CFP sample (m/E4) and sid2 were used as positive and negative controls respectively. Since ozone treatment was reported to increase ICS1 concentration in an earlier study (Ogawa et al 2007), the signals for ICS1 in WT samples were also expected to be strong in Agrisera antibody treated membrane. However, very light signal of ICS1 appeared for ozone treated WT plants suggesting that the antibody was not effective in detecting exogenous ICS1. Therefore, anti-ICS1 (Agrisera™) antibody needs to be tested again to get firm evidence of specificity for endogenous ICS1.

## 6.5 Perspectives

Transgenic ICS1-CFP plants can be regenerated by repeated cloning and transformation with proper troubleshooting which requires an elaborated time span. Once the parent homozygous line are established, molecular and physiological characterization of ICS1-CFP plants will be analyzed and by studying this, the function of ICS1 phosphorylation during prolonged photoperiod and exogenous stress will be revealed. On the other hand, activation pattern of MPK's in plants under different photoperiod and stress condition within detailed time course was studied, but the effect of day length and stress condition in activation pattern was not clear. In this circumstance, the experiment needs to be repeated with modifications and careful observation on plant growth, ozone treatment, and sampling.

# 7. Conclusion

In this study, our aim was to observe the function of ICS1 phosphorylation by molecular and physiological characterization of ICS1-CFP plants. By experimenting these plants, it would also be possible to study the role of ICS1 as an internal stress response regulator, and to observe how photoperiod influences its functionality. However, homozygous generation of ICS1-CFP plants could not be screened by selective propagation from parent ICS1-CFP plants. However, stress sensitivity was examined on candidate heterozygous (T2) ICS1-CFP plants grown in LD condition and the plants were found less sensitive to ozone. Stomata is an important factor in plants stress response since ozone enters into the plant through stomata. Moreover, stomata index analysis shows that photoperiod influences the stomata number in WT and SA knockout *sid2* plants. Since ozone treatment was not successful to establish a precise stress sensitivity pattern earlier, therefore, xanthine/xanthine oxidase treatment was applied in order to analyze stress sensitivity excluding stomata factor and observe different photoperiodic (LD and SD) impact on plants under stress. WT and *sid2* plants were subjected in this experiment to establish a distinguished stress sensitivity pattern under LD an SD condition. Although, *sid2* phenotype in both LD and SD conditions were found more sensitive to the treatments, there were no significant difference between the stress response patterns of this two phenotypes. Later, stress induced activation pattern of cellular MPK's (MPK3/MPK6) at different photoperiod (LD and SD) was also observed in a more detailed time course. Effect of stress was visible in both ozone and X-XO method, however impact of different day lengths or photoperiod on MPK's activation was unclear. As an additional part of this thesis, the specificity of the anti-ICS1 antibody provided by the company Agrisera$^{TM}$ was tested. The test results suggest that the antibody can detect complementary ICS1 from transgenic plant sample with strong signal but ineffective in detecting endogenous ICS1. In brief, some important aspects needs to be focused to achieve the goal of this study; an effective way out to generate transgenic ICS1-CFP plant lines within flexible time frame, troubleshooting for X-XO treatment and more experimental repetition with quantitative measurement regarding cellular MPK activation study.

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# Appendix I

Solutions and Buffers

| Solutions/Buffers | Content |
|---|---|
| LB medium | 10 g/l trypton<br>5 g/l yeast extract<br>10 g/l NaCl<br>Autoclave |
| 2X SDS extraction buffer | 100 mM Tris pH 6.8<br>200 mM DTT<br>4% SDS<br>20% Glycerol<br>1:100 cocktail of protease inhibitor |
| 4X SDS loading buffer | 200 mM Tris/HCl pH 6.8<br>400 mM DTT<br>8% SDS<br>0.4% bromophenole blue<br>40% glycerol |
| SDS solution (100 ml) for 10% resolving gel | 50 ml 1M Tris pH 8.8<br>1.35ml 10% SDS<br>Adjust to 100 ml with $dH_2O$ |
| SDS solution (100 ml) for 4.8% stacking gel | 13.6 ml 1 M Tris pH 6.8<br>1.14 ml 10% SDS<br>Adjust to 100 ml with dH2O |
| 10% separating gel (thickness 1.5mm) | 6 ml resolving gel buffer<br>2 ml 40% acrylamide<br>30 µl 10% APS<br>10 µl TEMED |
| 12% separating gel (thickness 1.5mm) | 5.6 ml resolving gel buffer<br>2.4 ml 40% acrylamide<br>30 µl 10% APS<br>10 µl TEMED |
| 4.8% stacking gel (thickness 1.5mm) | 4.4 ml stacking gel buffer<br>0.6 ml 40% acrylamide<br>24 µl 10% APS<br>10 µl TEMED |

| | |
|---|---|
| Lacus buffer | 25 mM Tris/HCl pH 7.8<br>10 mM MgCl2<br>15 mM EGTA<br>75 mM NaCl<br>1 mM DTT<br>1 mM NaF<br>0.5 mM Na3VO4<br>15 mM β-glycerophosphat<br>15 mM 4-nitrophenyl phosphate di(tris) salt<br>0.1% Tween 20<br>0.5 mM PMSF<br>5 µg/ml leupeptine<br>5 µg/ml aprotinin |
| 10X SDS running buffer | 15.2 g/l Tris base<br>72.1 g/l glycine<br>0.2 g/l SDS |
| Western transfer buffer | 50 mM Tris base<br>50 mM boris acid<br>Adjust pH to 8.3 |
| TBS-Tween solution | 150 mM NaCl<br>10 mM Tris/HCl pH 8.0<br>0.05% Tween 20 |
| Xanthine oxidase | 1 units/mg proteins<br>Suspension in 3M ammonium sulphate<br>10mM EDTA<br>pH 8.0 |

# Appendix II

**Analysis of Variance (ANOVA) tables for ion leakage experiments: Ozone and X/XO treatment**

A. ANOVA (Two-Factor Without Replication) of Ozone induced ion leakage experiment

$\alpha = 0.05$

| *SUMMARY* | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|
| m/WT 8 | 2 | 17,3836 | 8,6918 | 1,7019 |
| m/WT12 | 2 | 12,9811 | 6,4906 | 6,1380 |
| ICS1+TP E4 | 2 | 10,5323 | 5,2661 | 5,7794 |
| m/E4 | 2 | 13,1850 | 6,5925 | 11,1801 |
| m/D2 | 2 | 14,5824 | 7,2912 | 7,8105 |
| m/D5 | 2 | 11,1564 | 5,5782 | 5,2034 |
| | | | | |
| Control | 6 | 29,5827 | 4,9304 | 2,3060 |
| Ozone | 6 | 50,2381 | 8,3730 | 1,2150 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Rows | 15,3454 | 5 | 3,0691 | 6,7918 | 0,02776 | 5,05032906 |
| Columns | 35,5538 | 1 | 35,5538 | 78,6801 | 0,000303 | 6,60789097 |
| Error | 2,2594 | 5 | 0,4519 | | | |
| | | | | | | |
| Total | 53,1586 | 11 | | | | |

B.1. ANOVA (Two-Factor Without Replication) of X/XO treated ion leakage experiment
Long Day condition (LD)

α = 0.05

| *SUMMARY* | | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|---|
| WT control | | 4 | 61,5438 | 15,3860 | 56,8325 |
| WT 0.05 | | 4 | 128,9949 | 32,2487 | 325,6114 |
| WT 0.1 | | 4 | 160,0787 | 40,0197 | 436,0348 |
| sid2 control | | 4 | 85,7554 | 21,4389 | 128,3707 |
| sid2 0.05 | | 4 | 144,3570 | 36,0893 | 409,3231 |
| sid2 0.1 | | 4 | 157,1471 | 39,2868 | 468,5381 |
| | 0 | 6 | 58,0110 | 9,6685 | 8,9646 |
| | 1 | 6 | 155,6274 | 25,9379 | 69,2029 |
| | 3 | 6 | 241,4146 | 40,2358 | 175,8074 |
| | 5 | 6 | 282,8239 | 47,1373 | 259,3666 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Rows | 2049,2329 | 5 | 409,8466 | 11,8802 | 8,816E-05* | 2,9013 |
| Columns | 4956,6570 | 3 | 1652,2190 | 47,8928 | 6,437E-08* | 3,2874 |
| Error | 517,4745 | 15 | 34,4983 | | | |
| Total | 7523,3644 | 23 | | | | |

B.2 ANOVA (Two-Factor Without Replication) of X/XO treated ion leakage experiment
Short Day condition (SD)

α = 0.05

| *SUMMARY* | | *Count* | *Sum* | *Average* | *Variance* |
|---|---|---|---|---|---|
| WT control | | 4 | 50,6191 | 12,6548 | 56,6846 |
| WT 0.05 | | 4 | 76,4658 | 19,1164 | 165,6214 |
| WT 0.1 | | 4 | 92,7807 | 23,1952 | 240,6862 |
| sid2 control | | 4 | 41,9056 | 10,4764 | 40,7216 |
| sid2 0.05 | | 4 | 86,4302 | 21,6075 | 246,5596 |
| sid2 0.1 | | 4 | 106,1867 | 26,5467 | 371,9281 |
| | 0 | 6 | 21,2522 | 3,5420 | 0,6159 |
| | 1 | 6 | 82,1936 | 13,6989 | 13,3994 |
| | 3 | 6 | 156,6579 | 26,1096 | 89,6524 |
| | 5 | 6 | 194,2843 | 32,3807 | 129,1069 |

ANOVA

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Rows | 777,0047 | 5 | 155,4009 | 6,0253 | 0,002989608* | 2,9013 |
| Columns | 2979,7361 | 3 | 993,2454 | 38,5110 | 2,75638E-07* | 3,2874 |
| Error | 386,8683 | 15 | 25,7912 | | | |
| Total | 4143,6092 | 23 | | | | |